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# **ACEMBL Tool-Kits for High-Throughput Multigene Delivery and Expression in Prokaryotic and Eukaryotic Hosts**

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## **ABSTRACT**

Multicomponent biological systems perform a wide variety of functions and are crucially important for a broad range of critical health and disease states. A multitude of applications in contemporary molecular and synthetic biology rely on efficient, robust and flexible methods to assemble multicomponent DNA circuits as a prerequisite to recapitulate such biological systems *in vitro* and *in vivo*. Numerous functionalities need to be combined to allow for the controlled realization of information encoded in a defined DNA circuit. Much of biological function in cells is catalyzed by multiprotein machines typically made up of many subunits. Provision of these multiprotein complexes in the test-tube is a vital prerequisite to study their structure and function, to understand biology and to develop intervention strategies to correct malfunction in disease states. ACEMBL is a technology concept that specifically addresses the requirements of multicomponent DNA assembly into multigene constructs, for gene delivery and the production of multiprotein complexes in high-throughput. ACEMBL is applicable to prokaryotic and eukaryotic expression hosts, to accelerate basic and applied research and development. The ACEMBL concept, reagents, protocols and its potential are reviewed in this contribution.

## **1/ Complex challenge: Functional multigene assembly and delivery**

Multigene delivery into living organisms has taken to center stage in the synthetic biology era [1, 2]. This development has been catalyzed by the emergence of powerful technologies to precisely assemble DNA pieces representing functional modules into customized multifunctional DNA circuits. Recombinant DNA technology emerged half a century ago, when so-called ‘restriction factors’ were observed; which inhibited bacteriophage growth in bacteria, which turned out to be DNA endonucleases [3-6]. Around this time, DNA ligation was discovered as a basis of genetic recombination [7-9], leading to successful assembly of DNA fragments [10-14]. Since these ground-breaking discoveries, classical DNA cloning involved largely serial steps of cutting and pasting isolated fragments together by using restriction enzymes and DNA ligases, into functional DNA molecules (typically plasmids). Plasmids containing the DNA insert of choice then were delivered by transformation, transduction or transfection into prokaryotic or eukaryotic host cell organisms to exert their functions [15]. The advent of the polymerase chain reaction (PCR) enormously advanced the field [16], making DNA cloning commonplace in virtually all molecular biology laboratories worldwide. Today, DNA assembly has been further accelerated by new and powerful technologies, including ligation independent cloning methods (LIC, SLIC) [17, 18], circular polymerase extension cloning (CPEC) [19] and seamless ligation cloning extract (SliCE) [20], to name a few. For the assembly of very large fragments as precursors of entire synthetic genomes, specific cloning methods have been implemented [21]. Concomitantly, chemical DNA synthesis is being brought to perfection, considerably increasing the attainable size of DNA precursor fragments. These methods are at the core of synthetic biology, a vibrant field hailed as a game changer and poised to transform molecular biology and much of the life sciences [22-26].

Molecular cloning has been invaluable to study the structure and function of proteins by enabling heterologous expression. Elucidating the sequence content of entire genomes has made it possible to address the gene product repertoire - the proteome - of cells and organisms. Efficient DNA assembly methods to generate heterologous expression constructs have been implemented in concerted ‘omics’ efforts to analyze proteins system-wide, in high-throughput. Structural genomics consortia were established to determine atomic structures, seemingly in an industrial mode [27, 28]. Automation and robotics have become a prerogative; as a consequence, traditional cloning methodologies were progressively replaced by more advanced methods [1, 29-32]. A large number of vital functions in cells are mediated by multiprotein complexes composed of several to many subunits, and the function of a particular catalytic unit is often determined by its interaction partner(s). This has profound consequences for our understanding of the molecular mechanisms that are at the basis of biology. At the same time, this also imposes additional requirements on DNA assembly technology to support recombinant expression of complexes in high-throughput.

The ACEMBL technology was conceptualized to meet these requirements, to enable structural and functional “complexomics” research and discovery [31-34]. ACEMBL comprises recombination-based assembly of DNA elements into functional multigene expression constructs that can be rapidly permuted in a combinatorial fashion [33]. Originally, ACEMBL was developed for combinatorial multiprotein production in *E.coli* as a prokaryotic expression host [33]. Subsequently, efficient ACEMBL tool-kits have been developed also for multigene expression in eukaryotic hosts [34-37]. The integration of the ACEMBL technology in MultiBac, currently the lead technology for multiprotein complex production in insect cells, is described in a dedicated contribution of this issue [37]. The present overview therefore has as its focus the impact of ACEMBL on bacterial and mammalian multigene transfer applications.

## **2/ ACEMBL: Automated unrestricted DNA recombineering for multigene delivery**

Our knowledge of cellular processes have enormously advanced, brought about by an array of recent technological developments, notably in affinity purification, DNA sequencing, mass spectroscopy, yeast two-hybrid screens and computational approaches [38]. These technological developments compellingly validated the notion that virtually all essential cellular processes (DNA replication, transcription, translation, cell cycle regulation, intermediary metabolism, many more) are catalyzed by a highly coordinated network of protein-protein interactions, in which most proteins collaborate and function in the context of multiprotein complexes, underpinning the notion of ‘protein sociology’ in the cell [39].

Detailed structural and functional analysis is indispensable for elucidating the biological functions of these highly complex networks. Knowledge of molecular architectures can form the basis of intervention strategies, for example, to correct malfunction in disease states by supplying structure-based, custom-designed chemical compounds. Recapitulation of physiological interdependencies in the test-tube is a critical prerequisite for designing such compounds, and also for their preliminary validation by biochemical, biophysical and pharmacological means. Most multiprotein complexes, particularly those in humans, exist in (very) low endogenous amounts and are furthermore often heterogeneous in their composition, which is typically refractory to their extraction from native or cultured cell material. Heterogeneity in post-translational modifications, which may be essential for exerting the full activity of a given complex, can further limit the utility of material obtained from endogenous source.

Recombinant production offers solutions to these impediments, and a wide range of expression systems are available to produce proteins recombinantly in prokaryotic and eukaryotic hosts [31, 33, 34-45]. Recombinant expression systems share in common that one or several DNA segments encoding for proteins, protein domains or multicomponent protein

complexes are typically combined with DNA elements including DNAs that control transcription (promoters, terminators, others) and translation (ribosome binding sites, Shine-Dalgarno sequences, Kozak consensus sequences, enhancers, others) and inserted into a functional DNA module (plasmid, cosmid, artificial chromosome, genome, others). The resulting construct is then used to deliver the DNA segments of interest to host cell organisms by means of transformation, transfection or transduction.

The underlying technologies were perfected over the years to a level that they could be productively harnessed in ambitious, highly parallelized ‘omics’ programs aimed at genome- and proteome wide studies of proteins in many organisms including humans [28]. In these research undertakings, protein encoding genes are synthesized, manipulated, varied and delivered into recombinant expression hosts on an industrial scale to enable high-throughput structure determination, populating protein structure databases such as the protein data bank (PDB) with unmatched efficiency and breathtaking speed, ushering in a new age of protein structural and functional research.

Initially, these efforts were focused on single proteins, protein domains or small assemblies of two, maximally (rarely) three interactors. Our more recent understanding that the activity of a given protein catalyst can be decisively influenced by the (sometimes many) partners that arrange in multicomponent assemblies has to a certain degree challenged this minimalist approach. It is legitimate to postulate that, if proteins in cells act as parts of large and complex assemblies, then they should also be studied *in vitro* in the form of such large complexes, with a full complement of binding partners present. This approach offers opportunities and advantages, notably for drug discovery in pharma and biotech, where ‘being close(r) to physiological’ can be a tremendous asset. Evidently, however, it also complicates the experimental approach quite significantly, posing substantial technical challenges.

A multigene delivery system that affords to establish physiologically meaningful contexts *ex vivo* needs to be simple to use, robust, efficient and ideally compatible with automation and robotics, and readily accessible if similar breakthroughs for multiprotein complexes are to be achieved as have been successfully made already for single proteins and protein domains. We have taken advantage of more recent advances in DNA synthesis and molecular cloning technologies to develop ACEMBL, a technology concept that in our view successfully addresses these challenges [33]. ACEMBL exploits sequence and ligation independent multifragment cloning technology combined with site-specific multicomponent recombination for unrestricted assembly of multigene delivery constructs in a combinatorial fashion that is readily amenable to robotics [33, 46]. Affordable and efficient chemical synthesis methods of large DNAs as precursor molecules further potentiate the utility of ACEMBL for a broad range of applications.

### ***2.1/ ACEMBL DNA design***

The ACEMBL system utilizes a series of custom-designed vectors (called Acceptor or Donor, respectively) for multigene vector generation catalyzed by Cre-LoxP recombination [33, 34, 44, 46, 47]. All ACEMBL vectors are scratch-built, synthetic small plasmids (2-3 kilobases). Acceptor and Donor plasmids exclusively contain the minimal DNA elements absolutely required for protein expression and plasmid propagation, in addition to a set of DNA elements required for multigene assembly. In contrast to conventional expression plasmids including most commercial plasmids, these elements are directly juxtaposed, without intervening sequences devoid of functionality, giving rise to the smallest possible DNA molecules that propagate and can be used for multigene expression (Fig. 1).

ACEMBL plasmids contain common modules such as promoter / terminator and resistance marker. The Multiple Integration Element (MIE), adapted from a previously published polylinker [33], is tailored to support single or multiple gene insertions via



conventional restriction / ligation methods or, preferably, sequence and ligation independent cloning (SLIC) [33, 46] (Fig. 2). In addition, complementary homing endonuclease (HE) / BstXI site pairs are introduced for theoretically unlimited iterative gene insertions. We usually insert DNAs (genes of interest or fragments) that are chemically synthesized in the given format of choice, eliminating undesired restriction sites (including HE, BstXI) in the process.

There are two origins of replication in ACEMBL tool-kits; Acceptors contain a common *E.coli* origin of replication (BR322) and Donors contain a conditional origin of replication derived from phage R6K $\gamma$ . All plasmids contain a different resistance marker. Acceptors and Donors shown in Figure 1 contain elements that are specific for multigene delivery and expression in *E.coli* as a prokaryotic host. Similar Acceptors and Donors have been developed for multigene delivery and multiprotein complex expression in eukaryotic hosts, retaining the backbones but containing customized DNA elements (promoter / terminator pairs, gene integration sites, homologous recombination sequences, others) required in the respective eukaryotic host organisms (mammalian and insect cells).

## ***2.2/ Multigene assembly by Tandem Recombineering (TR)***

The SLIC reaction, in marked contrast to conventional cloning relying on restriction enzyme mediated digestion and ligation, can be readily scripted into a robotics routine [33]. ACEMBL Acceptor and Donor plasmids that contain one or several genes each are then concatamerized for multigene co-expression in a rapid and flexible fashion, by utilizing the LoxP imperfect inverted repeat sequences present on each plasmid, and the Cre recombinase which fuses LoxP sequences in a site-specific recombination reaction (Fig. 3) [48, 49]. Tandem Recombineering (TR) is the combination of SLIC-mediated gene integration and Cre-LoxP Acceptor-Donor fusion [46].

When educt DNAs containing single LoxP sites are subjected to Cre-LoxP recombination, only a small portion of educt DNAs are combined together, while the rest remain separate and co-exist with the fusion products. Acceptors contain a regular origin of replication (BR322), which enables their replication in regular *E. coli* strains (TOP10, OmniMAX, BL21, etc). In contrast, Donors contain a conditional origin of replication termed R6K $\gamma$  (the  $\gamma$  replication origin of the R6K plasmid) [50]. The replication of Donors requires the presence of the  $\pi$  protein (encoded by *pir* gene) in the host cell. Therefore, propagation and manipulation of all Donors has to be carried out in specific *E. coli* strains, which contain a *pir* gene inserted into their genome. Donors cannot replicate in a regular *E. coli* strain, which does not contain the *pir* gene (i.e. *pir*-negative), unless fused with an Acceptor with a regular origin of replication. Thus, the recombination of Acceptors and Donors can be exploited for specific selection of desired fusion products.

A single Acceptor could be recombined in a single Cre-LoxP reaction with a theoretically unlimited number of Donors, with one to several expression cassettes on each Donor and Acceptor. Pragmatically, we use one Acceptor and up to three Donors to generate multigene constructs for heterologous expression. Due to the equilibrium nature of the Cre-LoxP reaction, the recombination reaction products are a mixture of all possible fusions from two or more educts, including Acceptor-Acceptor, Acceptor-Donor, and Donor-Donor fusions. Since excision is favored, fusion products containing increasing numbers of educts are present in decreasing amounts. All fusion products and also the single educt plasmids are quasi bar-coded by their characteristic resistance marker combinations (Fig. 4), as all plasmids of the system have a different resistance marker. After transformation into regular *E. coli* strains (*pir*-negative background), all unwanted Donors and Donor-Donor fusions are eliminated since their conditional origins are inactive in *pir*-negative *E. coli* strains, while the desired Acceptor-Donor fusions are selected by challenging with corresponding combinations

of antibiotics (Fig. 4). This enables the tailored generation of multigene vectors expressing a complete protein complex as well as subsets of its subunits, in a single Cre-LoxP reaction. This combinatorial approach is instrumental for investigating the hierarchical assembly of multiprotein complexes, the biological functions of specific subunit(s) or their combinations, as well as the integration of putative subunit isoforms into a multiprotein complex of choice [31]. Thus selected arrays of fusion plasmids can then be used for gene delivery into expression host cells, optionally in high-throughput.

Subsequent to antibiotic challenge, fusion plasmids can (and probably should) be verified by restriction mapping. For example, transformants might contain fusion products harboring more than one copy of a particular educt vector. This can be potentially detrimental by causing expression level imbalance between subunits due to the increase in copy number of the gene(s) present on the particular educt. On the other hand, this could also be used to the benefit of the expression experiment. When a certain gene of interest is expressed at lower levels as compared to other genes in a multigene expression experiment, it can be helpful to incorporate an additional copy of the corresponding educt plasmid, and/or to place the same gene in several copies on one or more educt plasmids prior to the Cre-LoxP fusion reaction.

When more than two educt vectors are subjected to Cre-LoxP recombination, their incorporations are stochastic and thus lead to sequence variations in the fusion plasmids depending on the assembling orders of educt vectors (Fig. 5). The number of possible fusion plasmids ( $P_n$ ) containing  $n$  educt vectors (each as a single copy) is given by the formula of circular permutation:  $P_n = (n-1)!$ . For example, a fusion plasmid containing one acceptor and three donors ( $n=4$ ) has  $P_4 = 3! = 6$  possible variants (Fig. 5). From our experience, the order of assembly of educts in a multifusion plasmid apparently does not prejudice the success of a complex expression experiment. Nonetheless, good practice requires verifying the order of

assembly of educts in the multifusion plasmid as a quality control step. Therefore, the exact DNA sequences of all possible fusion variants are required for verification and selection by restriction digestions. To facilitate the *in silico* generation of DNA sequences of all possible fusion variants, we programmed a software application, Cre-ACEMBLER [51, 52].

### ***2.3/ Cre-ACEMBLER software***

Cre-ACEMBLER was programmed in Python and runs on Windows, Linux, and MacOS operating systems. Cre-ACEMBLER displays sequence data in an application window, showing the sequence as plain text. Simple manipulations can be done using cut, copy and paste functions. Sequence data can be read from and written to files in various formats, including FASTA and GenBank.

To perform *in silico* Cre recombinations, all educt plasmid sequences have to be opened in Cre-ACEMBLER. Activating the “Cre” button starts an assistant dialogue guiding through the recombination in three steps: (1) Acceptor plasmid sequence is selected among all open sequences; (2) Donor plasmid sequences are selected; (3) Adjustment of the desired copy numbers of each individual plasmid. Each possible product sequence is then generated and displayed in a new window. Product sequences can then be saved to files and analyzed using other software, e.g. ApE [53] or Vector NTI [54]. Prerequisites to be fulfilled by Cre-ACEMBLER were ease of use, compatibility with a broad range of operating systems and interoperability with other software. No central processing unit (CPU)-intensive work is done by Cre-ACEMBLER, thus an interpreted programming language could be chosen without risking performance limitations. Therefore, Cre-ACEMBLER was developed in Python [56], using the Python bindings of GTK+ [57, 58] for the graphical user interface, and the Biopython [59] library for sequence data manipulations. Using Python and GTK+ allows Cre-ACEMBLER to run on Windows, Linux and MacOS operating systems, and possibly

others. The Biopython library allows reading and writing sequence data in various file formats, providing good interoperability with other software.

It is of advantage for the *in silico* Cre recombination if LoxP sites in all input (educt) sequences are in the same orientation, and if the linear representation of each input sequence starts with the LoxP site. Therefore, all input sequences are normalized prior to recombination, by generating the reverse-complement of input sequences if required, and by linearizing all sequences immediately 5' of the LoxP site. All input sequences are then indexed numerically, making sure that identical input sequences get the same index. Lists representing all possible permutations of the order of the indices are computed, and redundant solutions (if considering circular arrangement) are eliminated, thus yielding index lists representing only unique circular permutations. Fusion plasmid sequences are then generated from these index lists by appending the normalized input sequences corresponding to the indices, in the order given in these lists.

A challenge arising from the linear representation of circular sequences is to identify permutations which are redundant if circular arrangement is considered. In order to make the lists representing different circular arrangements comparable, a linearization algorithm had to be found which transforms a linear representation with a random starting point reliably into a linear representation with a defined starting point. To accomplish this, the lowest index in the lists is taken as a potential starting point for linearization. If several instances of this lowest index are present in the list, each instance is credited a score according to the subsequent indices in the list. The instance that is followed by the highest count of lowest indices gets the highest score, and the list is rearranged such that this instance becomes the first entry. Lists transformed in this way can then simply be compared using Python's equality operator, so that redundant solutions can be identified and eliminated.

Cre-ACEMBLER has proven to be a valuable, robust tool in extensive testing by users of the Eukaryotic Expression Facility (EEF) at EMBL Grenoble, proving the reliability of the algorithms described above. Cre-ACEMBLER is freely available for download [51]. A Cre-ACEMBLER User Manual is likewise available on-line [52].

### **3/ACEMBL applications**

The ACEMBL system was first introduced for robotized production of multiprotein complexes in high-throughput [33]. Subsequently, the ACEMBL pipeline was extended to eukaryotic expression systems (Fig. 6) in order to produce functional eukaryotic protein complexes requiring the authentic processing and post-translational machinery provided by eukaryotic hosts [31]. Multifusion plasmids generated from Cre-LoxP reactions are utilized by the ACEMBL-derived MultiMam system to facilitate simultaneous multigene introduction into mammalian cells [35, 36] (see also 3.2). The MultiBac baculovirus / insect cell system has been upgraded for robotics by incorporating ACEMBL DNA modules (MIE and HE / BstXI sites) for automatable and theoretically unlimited multigene insertion into a baculoviral genome for protein co-expression in insect cells [34] (see also Chapter on MultiBac by Sari and co-workers in this issue). Selected examples of ACEMBL applications are highlighted in the following.

#### **3.1/ ACEMBLing DNA for structural and molecular biology**

ACEMBL has been used successfully for a variety of applications in structural and molecular biology. Numerous multisubunit complexes, including soluble multiprotein complexes, protein-RNA complexes and multimeric membrane protein complexes have been produced successfully by ACEMBL [33, 44, 60-64]. Examples include the prokaryotic signal recognition particle, SRP, the catalytic cycle of which is being studied by cryo-electron microscopy and biochemical means [60-62]. A particular highlight is the prokaryotic holo-

translocon complex (HTL), a seven subunit transmembrane multiprotein assembly consisting of the heterotrimeric core translocon, SecYEG, and its accessory proteins SecD, SecE, YidC and YajC. HTL is a long elusive complex that was, for the first time, successfully produced recombinantly by ACEMBL [63, 64]. HTL catalyzes the transport of protein substrates through and into membranes, making use of the proton motive force (PMF) [63]. Moreover, ACEMBL was applied to reveal the substrate specificity for interferon-stimulated gene 15 by ubiquitin-specific protease 18 [65]. Many research laboratories have already obtained ACEMBL reagents, and ACEMBL systems are in the process of being integrated into structural genomics pipelines. We expect in the coming years numerous more exploits brought about by our multigene delivery technologies, and we anticipate productive synergies with other multigene recombineering tools, to deconvolute internal redundancy and explore functional structure in complex biological systems [66-69].

### **3.2/ Highly efficient multigene delivery in mammalian cells**

We implemented TR to facilitate rapid generation of multicomponent gene expression circuits from Acceptors and Donors containing mammalian cell active promoters [35, 36] (Fig. 6). These multicomponent circuits are used for efficient multigene delivery in mammalian cells, resulting in homogeneous cell populations [35]. Such results could not be obtained previously by classical methods relying on co-transfection of plasmids modules. Using fluorescently labelled proteins to visualize mammalian cell compartments, their substructures and contents is a common technology in cell biology and pharmacological applications. Homogeneous cell populations are a prerequisite for monitoring perturbations of cell states, biological processes, metabolic pathways, signaling cascades and the effect of additives, for example in high-content screening. The utility of the TR approach to generate homogeneous cell populations by multigene delivery of fluorescently labelled proteins was compellingly demonstrated using pig cardiac endothelial cells that expressed five different

proteins, delivered by a TR construct fitted with mammalian cell active promoters [35]. A constant relationship between expression levels of the proteins at the level of individual cells was demonstrated [35]. Moreover, this approach was applied to analyze the localization of epidermal growth factor receptor (EGFR) with Ran GTPases in endosomal trafficking, and to demonstrate how Neuropilin-1 promotes VEGFR-2 trafficking through Rab11 vesicles, thereby specifying signal output [35, 70].

We anticipate that a wide range of applications will benefit from a synchronized delivery of multiple genes. Our innovative approach has the potential to facilitate the production of multicomponent protein drugs including next-generation vaccine candidates such as virus-like particles. Multiplexed labelling of living cells, protein-protein interaction studies, the construction of designed gene regulatory circuits and entire synthetic signalling cascades are further active research and development fields that could benefit from ACEMBL technologies.

#### **4/ Metabolic engineering**

Metabolic engineering is emerging as an overarching concept subsuming a collection of methods and concepts for re-directing, improving or modifying cellular and organismal biochemical pathways, with the goal of generating novel qualities. At the core of synthetic biology, metabolic engineering has been defined as “the purposeful modification of cellular activities with the aim of strain improvement” [71]. A purpose is to achieve an overall higher productivity and superior quality of scientifically or commercially interesting molecules in research and development, and in industrial settings. These can include proteins, protein complexes, nucleic acids, biochemicals and metabolites that normally do not accumulate to a significant degree or sufficient quality, and would otherwise have to be chemically synthesized or extracted from natural sources. Moreover, complex chemical structures, for



instance with multiple chiral centers, often are easier produced in engineered microorganisms or cells, at lower cost.

Researchers wish to tweak the host which is the organismal “factory”, by altering its biological traits, to produce modified or new substances [72]. Such refinements require considerable genetic engineering for custom-design of entire regulatory circuits and metabolic pathways and their efficient delivery into the host organism. Concurrently, ‘negative’ factors need to be removed, which would otherwise be detrimental to achieving the desired product yields and quality improvements. For example, production strains may need to be made more resilient to demands incurred by (multi)protein overexpression [73]. Physiological knowledge of the pathways under investigation, choice of the right production organism, information from metabolic flux modelling and bioprocess development need to be considered and addressed in the design of the synthetic multifunctional DNA circuits to be delivered [74]. This can then be exploited for the improved production, up to fermenter scale, of protein therapeutics such as monoclonal antibodies, commodity chemicals such as vitamins or rare amino acids, valuable metabolites, biomolecules eliciting fragrances and flavors, rare natural (medicinal) compounds (such as artemisinin and taxol) or even biofuel production [74-78]. While some constraints can be overcome by optimizing culture conditions, others can more successfully be tackled by modifying defined metabolic pathways. A thorough knowledge of the cellular biochemistry in conjunction with new and powerful recombinant DNA technology now allows “to rationally modify and design metabolic pathways, proteins, and even whole organisms.” [78].

Biosynthetic pathways can now be (re)constructed from scratch and adapted to a host organism to either replace or complement endogenous pathways [75, 79]. Genetic modifications involved include for example plugging in appropriate regulatory elements into the plasmid constructs, optimizing codon usage or transcription factor activity, and tuning the

effects of intergenic regions. At the same time, endogenous pathways can be shut down or reduced [80] to optimize the balance between heterologous and endogenous biochemical activities [79]. Side effects or roadblocks encountered can be ameliorated or removed by multiple rounds of engineering [79, 81, 82].

ACEMBL tool-kits, due to unmatched flexibility and robustness, in our view may be optimally suited to address these manifold requirements for building multifunctional heterologous expression constructs, predominantly to equip *E.coli*, insect and mammalian cells with multiple genes and functionalities, combinatorially arranged by TR in multicomponent DNA regulatory circuits. An advantage of ACEMBL is that individual (sets of) components can be distributed on several plasmid modules (Acceptors and multiple Donors) and recombined as desired. Furthermore, individual (sets of) components can be flexibly modified without compromising other (sets of) components, and new components introduced if required. Moreover, gene regulatory elements including promoters and terminators can be altered or tuned with ease, and adapted to the host organism and the specific requirements of the target molecule(s).

## 5/ Conclusion

The ACEMBL technology concept was originally conceived to synergistically address two sets of requirements. On the one hand, we intended to create technologies that assist in making hitherto inaccessible target molecules, in particular multiprotein complexes, amenable to high-resolution structural and functional analysis as a prerequisite to better understand their cellular activities, and to enable their modulation for example if malfunction occurs in disease states. On the other hand, we wanted our technologies to be sufficiently robust to facilitate automation and robotics, to harness the benefits of parallelized workflows that already have been established for high-throughput applications with remarkable success. ACEMBL fulfills these requirements, and we are hopeful that the methods we developed will contribute significantly to the system-wide elucidation of the protein ‘complexome’ of cells and organisms, in health and disease. Currently, ACEMBL reagents are available for multigene delivery and heterologous expression in *E.coli*, mammalian cells and insect cells as hosts, and further systems targeting other important organismal factories are forthcoming. Moreover, beyond heterologous protein complex production, ACEMBL holds significant promise to catalyze synthetic biology approaches which are at the forefront of current biology, by enabling multiplexed assembly of synthetic multicomponent DNA constructions for highly efficient multigene delivery, in prokaryotic and eukaryotic hosts, for a wide range of applications.

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## Figures Legends

**Figure 1. ACEMBL technology concept.** (A) Acceptor and Donor plasmids are shown in a schematic view (top). The examples shown here are used for multigene delivery in *E.coli* as an expression host. Acceptor and Donor vectors contain a LoxP sequence and an identical Multiple Integration Element (MIE). Promoters (T7 or *lac*), corresponding terminators and homing endonuclease (HE) sites (blue strike-through box, Acceptors: I-CeuI; Donors: PI-SceI) and matching BstXI sites (small blue squares) are indicated. Origins of replication (Acceptors: BR322; Donors: R6K $\gamma$ ) are shown. Ap: Ampicillin, Tet: Tetracycline, Cm: Chloramphenicol, Kn: Kanamycin, Sp: Spectinomycin. The Multiple Integration Element (MIE) is specific for expression in a prokaryotic host and supports assembly of polycistrons encoding for several genes controlled by a single pair of promoter and terminator. (B) Outline of the method (adapted from [33]).

**Figure 2. Gene insertion into ACEMBL Acceptors and Donors by SLIC.** Gene insertion into ACEMBL plasmids by sequence and ligation independent cloning (SLIC) is shown in a schematic representation. Primer DNA oligonucleotides used for PCR are shown as thin bars with arrows. RBS stands for ribosome binding site. 5' denotes the five-prime end. Regions of homology in the Multiple Integration Element (MIE) are shown as boxes filled in gray. Single gene integration is shown on the left. Multigene integration yielding a polycistron is depicted on the right. PCR stands for polymerase chain reaction. Exonuclease treatment is conveniently performed by T4 DNA polymerase in the absence of deoxyribonucleotide triphosphates (dNTPs) (adapted from [33]).

**Figure 3. Cre-LoxP fusion reaction.** Mechanism of Cre-mediated DNA fusion is shown in a schematic representation. Cre enzyme (shape filled in gray) recognizes LoxP sites (marked by dashed lines and arrow) present on DNA molecules and fuses them in an equilibrium reaction favoring excision (left). The sequence of the LoxP imperfect inverted repeat is

displayed (top, right). Cre-LoxP mediated fusion is a one-step reaction requiring a simple protocol that can be automated. The structure of four copies of Cre enzyme bound to a Holliday junction reaction intermediate is shown in the inset (adapted from [49]).

**Figure 4. ACEMBL combinatorics.** Dynamic assembly (Cre) and disassembly (De-Cre) of Acceptor and Donor plasmids by Cre-LoxP reaction is shown schematically (top). LoxP sites are drawn as red circles, resistance markers and origins of replication are colored as above (Fig. 1). White thick arrows denote expression cassettes. AD stands for Acceptor-Donor fusion. ADD stands for Acceptor-Donor-Donor fusion. Not all possible fusion products are shown for clarity. Levels of multiresistance for product selection are indicated (top, right). All reactions occur in a single Eppendorf tube. Fusion products co-exist with educts. Productive fusion products are selected using (multi)antibiotic challenge, for example on a 96-well micro-titer plate (bottom). Desired Acceptor-Donor fusions are identified according to their resistance marker ‘bar-code’. Color-coding of antibiotics is listed (bottom, right). LB stands for Luria-Bertani / lysogeny broth.

**Figure 5. Acceptor-Donor fusion arrays.** Variants of possible multifusion plasmids are depicted, containing two (top row), three (middle row), or four (bottom row) educt plasmids (Acceptor, Donors), each as a single copy. Box filled in red denotes Acceptor (A), Boxes filled in green, blue and purple denote three Donors (D1, D2 and D3, respectively). The linear order (starting with A for simplicity) of educts in each (circular) multifusion plasmid is indicated below the corresponding plasmid map. The number of educt vectors and compositions are indicated (right).

**Figure 6. ACEMBL tool-kits (as of 2014).** Prokaryotic and eukaryotic expression systems derived from ACEMBL technology for multiprotein co-expression (adapted from [31]). Note that initially, ACEMBL referred to the *E.coli* system. We have now named the individual

ACEMBL systems MultiColi for *E.coli*, MultiMam for mammalian and MultiBac for baculovirus/insect cell expression.

## Figures

Figure 1. ACEMBL technology concept.

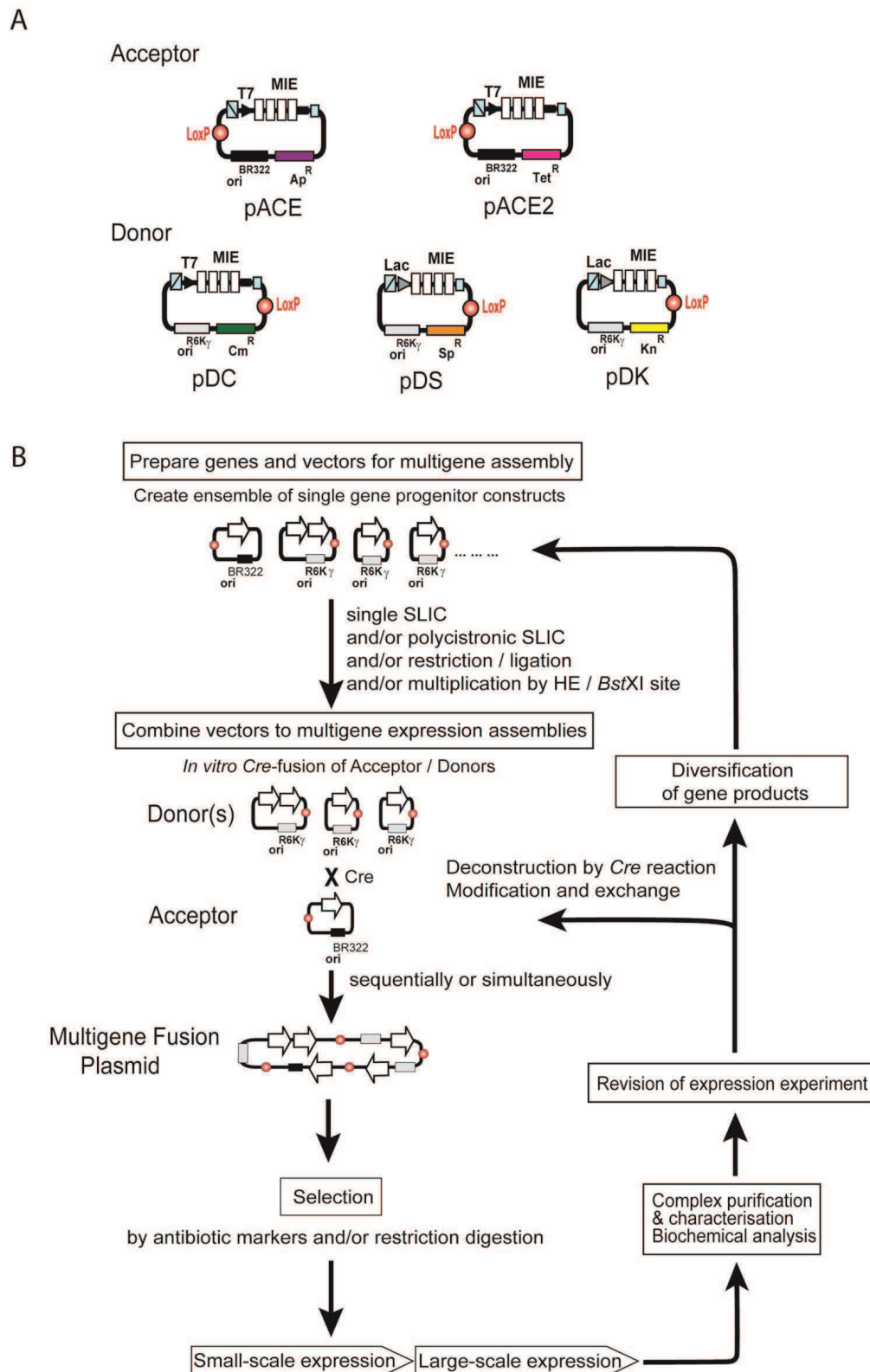


Figure 2. Gene integration in ACEMBL Acceptors and Donors by SLIC.

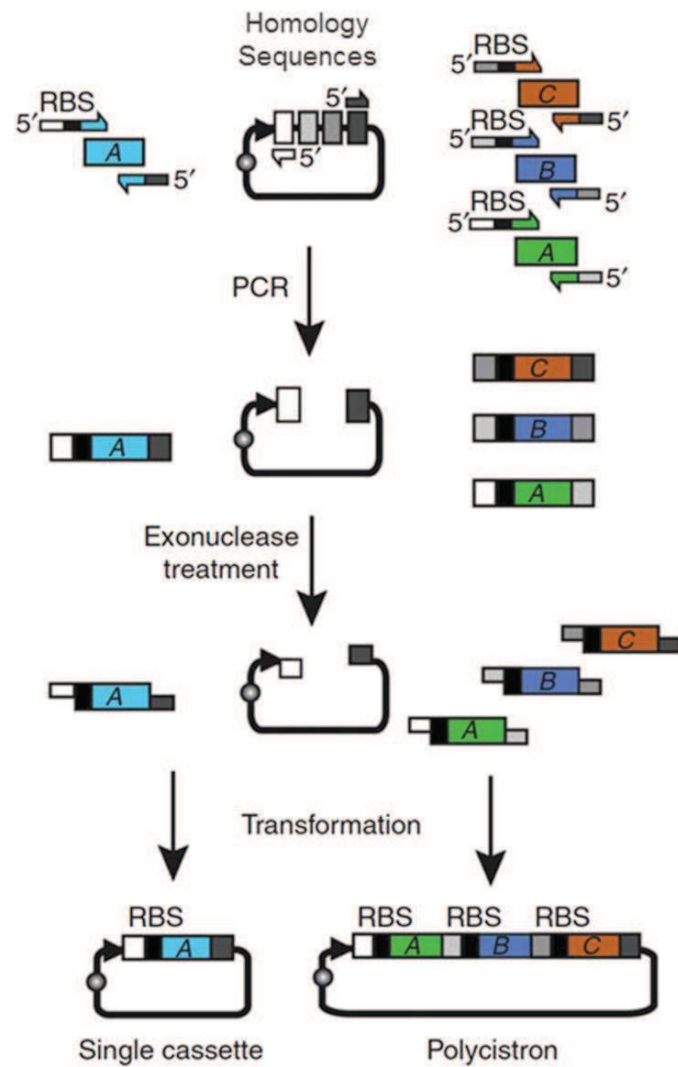


Figure 3. Cre-LoxP fusion reaction.

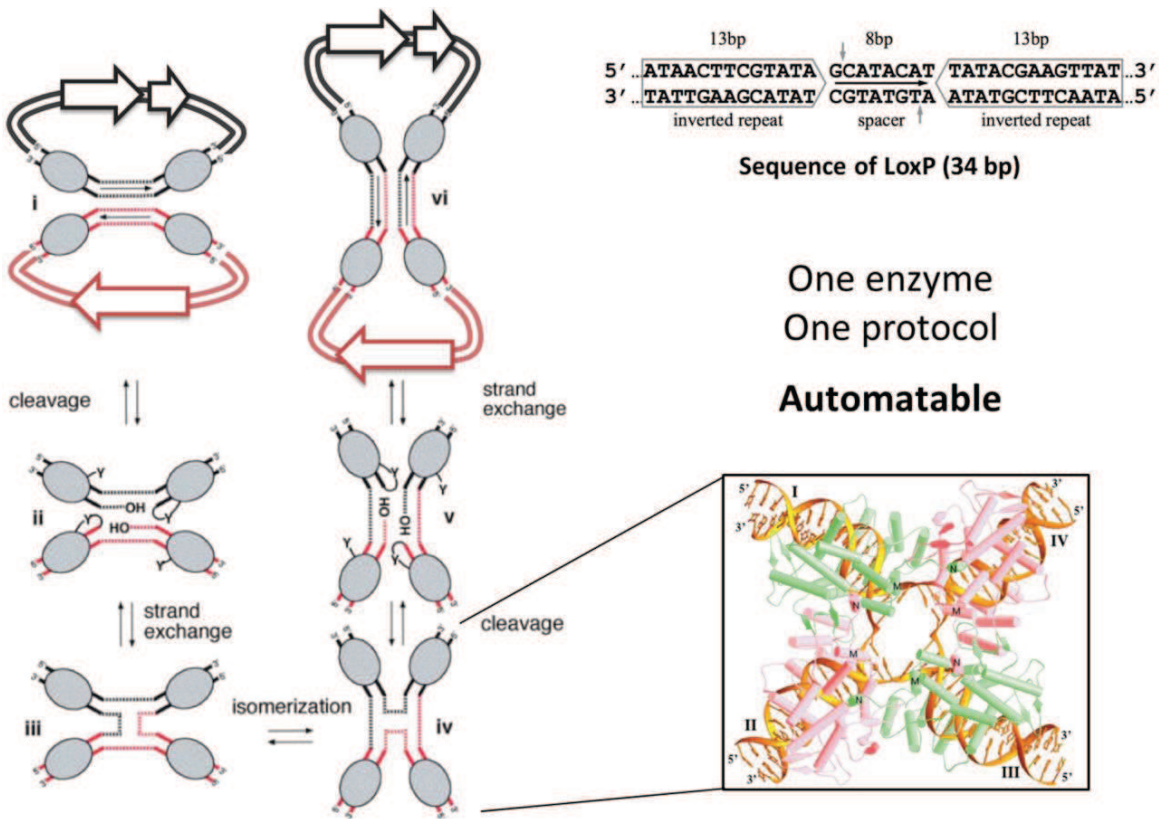


Figure 4: ACEMBL combinatorics.

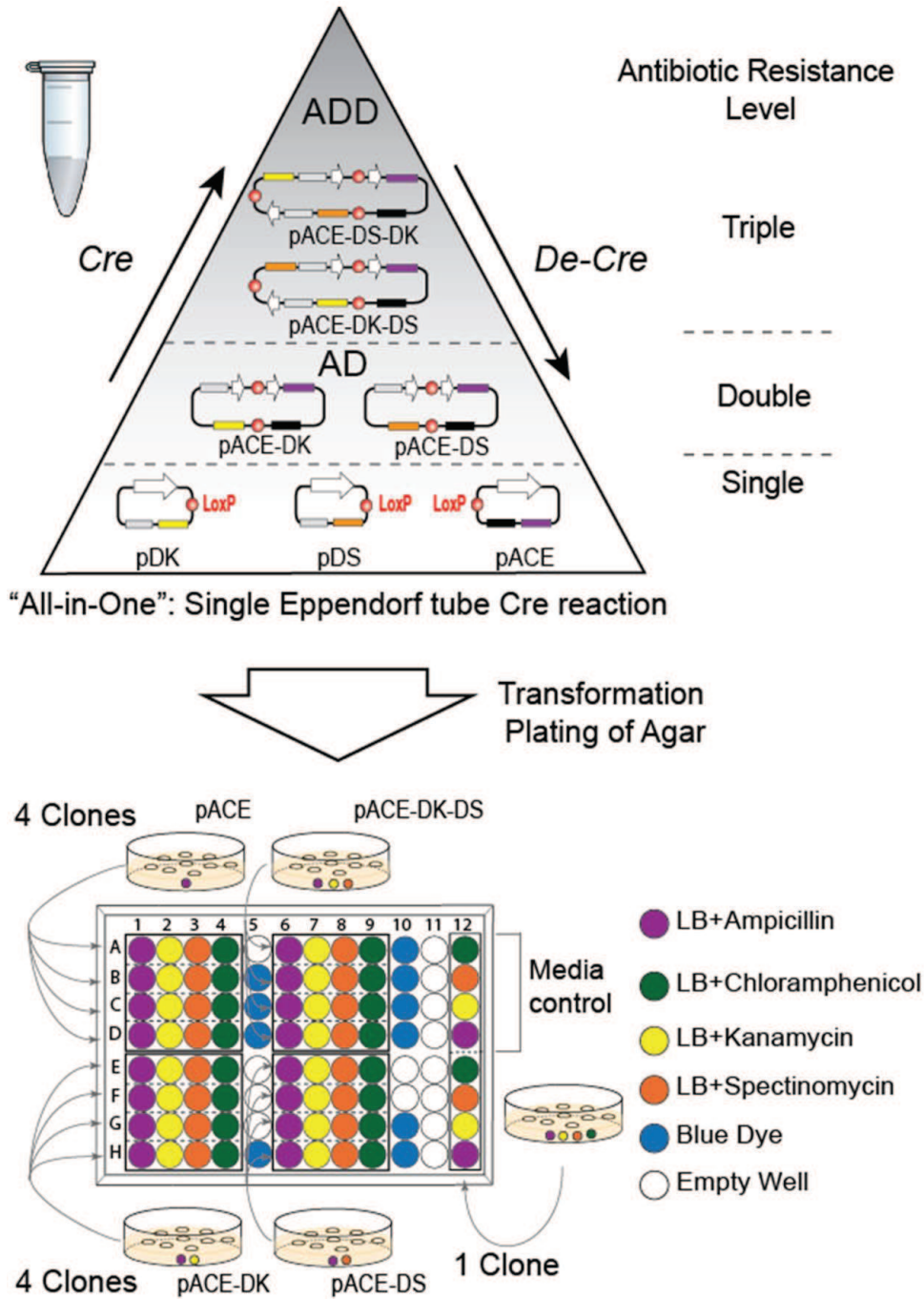




Figure 5. Acceptor-Donor fusion arrays.

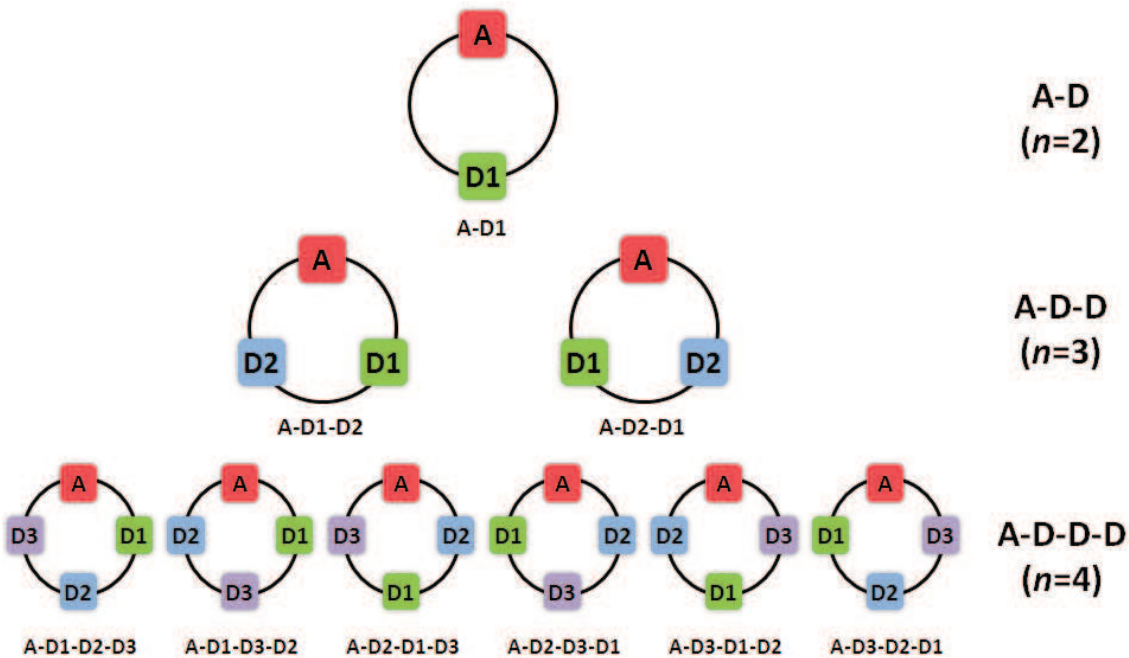


Figure 6. ACEMBL tool-kits (as of 2014).

